

# Obligatory participation of macrophages in an angiopoietin 2-mediated cell death switch

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Macrophages have a critical function in the recognition and engulfment of dead cells. In some settings, macrophages also actively signal programmed cell death. Here we show that during developmentally scheduled vascular regression, resident macrophages are an obligatory participant in a signaling switch that favors death over survival. This switch occurs when the signaling ligand angiopoietin 2 has the dual effect of suppressing survival signaling in vascular endothelial cells (VECs) and stimulating Wnt ligand production by macrophages. In response to the Wnt ligand, VECs enter the cell cycle and in the absence of survival signals, die from G1 phase of the cell cycle. We propose that this mechanism represents an adaptation to ensure that the macrophage and its disposal capability are on hand when cell death occurs.

**KEY WORDS:** Macrophage, Angiopoietin, Wnt, Programmed cell death, Vascular regression, Cell cycle

## INTRODUCTION

Macrophages have a critical function in tissue homeostasis because they can rapidly recognize and engulf dead cells (Savill et al., 2002). Rapid identification and engulfment of apoptotic cells by macrophages prevents the release of pro-inflammatory intracellular components that when chronically released can be deleterious. Indeed, recognition of apoptotic cells by macrophages results in the release of anti-inflammatory agents including TGF $\beta$  (Savill et al., 2002; Lucas et al., 2003). In many circumstances where macrophages encounter apoptotic cells, the macrophage is believed to have a after-the-fact role in disposal and immune modulation.

There is emerging evidence to suggest that in some cases, macrophages play an active role in inducing programmed cell death. This was first appreciated after macrophage ablation experiments resulted in the persistence of viable cells in ocular vessel networks that failed to undergo scheduled regression (Lang and Bishop, 1993). The proposal that macrophages can actively signal cell death has received support from a number of systems including *C. elegans*, where caspase (CED-3) partial function combined with recognition pathway mutants demonstrate that recognition is a back-up stimulus for apoptosis (Hoepfner et al., 2001; Reddien et al., 2001). Although there is currently no mechanistic parallel apparent, discovery of macrophage-induced (Lang and Bishop, 1993; Diez-Roux and Lang, 1997) and phagocyte-enhanced (Hoepfner et al., 2001; Reddien et al., 2001) apoptosis in mice and worms may suggest an activity functionally conserved in metazoans.

Signaling mechanisms accounting for macrophage-induced cell death are now being described. In the developing chick retina, it has been demonstrated that macrophage-related microglia are a source

of nerve growth factor (NGF) that causes neurons to die (Frade et al., 1996), and in mice, microglia promote programmed cell death of Purkinje cells through production of superoxide ions (Marin-Teva et al., 2004; Mallat et al., 2005). In the mouse eye the canonical Wnt pathway (Logan and Nusse, 2004) is crucial for scheduled vascular regression. In this system, resident macrophages produce Wnt7b and through close contact with vascular endothelial cells (VECs) signal cell death (Lobov et al., 2005).

The angiopoietin signaling system plays a key role in the regulation of angiogenesis, vascular homeostasis and vascular regression (Yancopoulos et al., 2000). One angiopoietin receptor is the conventional tyrosine kinase receptor Tie2 (Dumont et al., 1994). Expression of Tie2 is largely restricted to endothelial cells but can also be found in haematopoietic cells and a subset of tumor-associated monocytes and eosinophils (Iwama et al., 1993; Dumont et al., 1994; Feistritzer et al., 2004; De Palma et al., 2005). More recent work has indicated that integrins can serve as alternative receptors for the angiopoietins (Carlson et al., 2001) and that integrins and Tie2 can form a complex (Cascone et al., 2005).

Angiopoietin 1 (Ang1; also known as Angpt1 – Mouse Genome Informatics) and angiopoietin 2 (Ang2; also known as Angpt2) are the two best characterized Tie2 ligands. Ang1 (Suri et al., 1996) is an agonist and elicits many responses including cell-survival through the PI3-kinase-Akt signaling pathway (Datta et al., 1999; Peters et al., 2004). Under some (Gale et al., 2002; Lobov et al., 2002) but not all (Kim et al., 2000b; Gale et al., 2002) circumstances, Ang2 (Maisonpierre et al., 1997) is a signaling antagonist and inhibits the action of Ang1. As an antagonist of the PI 3-kinase-Akt signaling pathway, Ang2 activity is synonymous with survival signal withdrawal. Ang2 promotes vessel destabilization (Maisonpierre et al., 1997) and, in the presence of vascular endothelial cell growth factor (VEGF), is an important angiogenic stimulus (Yancopoulos et al., 2000; Lobov et al., 2002). Under conditions where VEGF signaling is inhibited or absent, the action of Ang2 in destabilizing vessels can lead to cell death and capillary regression (Yancopoulos et al., 2000; Lobov et al., 2002).

The hyaloid vessels of the eye serve as a model system to further our understanding of the mechanisms of vascular regression (Lang and Bishop, 1993; Diez-Roux and Lang, 1997; Diez-Roux et al.,

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1999; Ito and Yoshioka, 1999; Meeson et al., 1999; Ylikarppa et al., 2003; Ohlmann et al., 2004; Lobov et al., 2005). In the current study we sought to explain the mechanisms by which hyaloid VECs undergo apoptosis and examined the possibility of cooperation between the Wnt and angiopoietin pathways. We show that during scheduled vascular regression, macrophages are an obligatory participant in a signaling switch that favors death over survival. This switch occurs when Ang2 has the dual effect of suppressing PI 3-kinase-Akt survival signaling in VECs and stimulating Wnt7b production in macrophages. In response to Wnt7b, VECs enter the cell cycle and die in the G1 phase of the cell cycle due to Ang2-mediated withdrawal of survival signals. We propose that close coupling of macrophage function to the cell death program is an adaptation to ensure that a 'professional' phagocyte is on hand when cell death occurs.

## MATERIALS AND METHODS

### Mouse genotyping

Genotyping of *Lrp5<sup>lacZ</sup>* (Kato et al., 2002), *Wnt7b<sup>lacZ</sup>* (Lobov et al., 2005), *Wnt7b<sup>dl</sup>* (Lobov et al., 2005), *Ang2<sup>lacZ</sup>* (Gale et al., 2002), *VE-cadherin:rtTa* (Sun et al., 2005), *TET:myrAkt* (Sun et al., 2005) and *TOPGAL* (DasGupta and Fuchs, 1999) mice were performed as previously described.

### Dissections, immunostaining and imaging

Hyaloid vessel preparations were generated as previously described (Lobov et al., 2005) with the exception that dissections were performed without gelatin. X-Gal staining was done according to established protocols (Shu et al., 2002). Indirect immunofluorescent staining and BrdU labeling were performed as previously described (Diez-Roux et al., 1999). Primary antibodies were as follows: anti-BrdU (1:100, Dako), anti- $\beta$ -catenin (1:50, BD Transduction). Secondary antibodies labeled with Alexa fluorochromes (Molecular Probes) were used at a 1:500 dilution. TUNEL labeling of apoptotic cells was performed using the In Situ Cell Death Detection Kit (Roche Applied Science).

### Cell culture experiments

Microvascular endothelial cells (MVECs; Cambrex) from skin were grown to 60–70% confluency, serum starved overnight and stimulated with Ang1 (500 ng/ml) and/or Wnt3a (250 ng/ml) in combination with the Akt inhibitor SH6 (Kozikowski et al., 2003; Castillo et al., 2004) (10  $\mu$ M) or the PI 3-kinase inhibitor wortmannin (250 nM) for 4 hours or 12 hours and labeled for  $\beta$ -catenin.

### Intravitreal injection

Intravitreal injection was performed with glass needles pulled on a model P87 flaming-pipette puller and beveled with a K. T. Brown Type model BV-10 diamond beveller (Sutter Instrument Company, Novato, CA). Materials injected included 120 nl of 10  $\mu$ M SH6 (Alexis Biochemicals) and 120 nl of 50  $\mu$ M Ang1 or Ang2 (R&D Systems).

### Laser capture microdissection

Macrophages were captured from whole-mount hyaloid vessels of P3 CD1 mice, injected with Ang2 intravitreally (see above) using the Veritas Microdissection System. Control eyes were sham injected with PBS. For each experiment, 5–7 ng/ml of RNA was used.

### RT-PCR

RNA from dissected hyaloid vessel preparations was purified using the Qiagen MicroEasy RNA Isolation Kit. Subsequent RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen). The following primers (shown 5' to 3') were used:

*Gapdh*: forward (F), ACTCCACTCACGGCAAATTC; reverse (R), CACATTGGGGGTAGGAACAC.

*Ang1*: F1, AGGCTTGGTTTCTCGTCAGA; R1, TCTGCACAGTCTCGAAATGG.

*Wnt7b*: F, AAGAACTCCGAGTAGGGAGTCG; R, TGCGTTGTACTTCTCCTTGAGC.

*Wnt7b*: 2nd round F, CCGAGTAGGGAGTCGAGAGG; R, CACACCGTGACACTTACATTCC.

### Statistical analysis

Vessel number was quantified using established methods (Ito and Yoshioka, 1999). At least four hyaloid vessel preparations were quantified for each experiment. All data are presented with standard error bars. The Student's *t*-test and one-way ANOVA with Tukey's test were used to assess statistical significance.

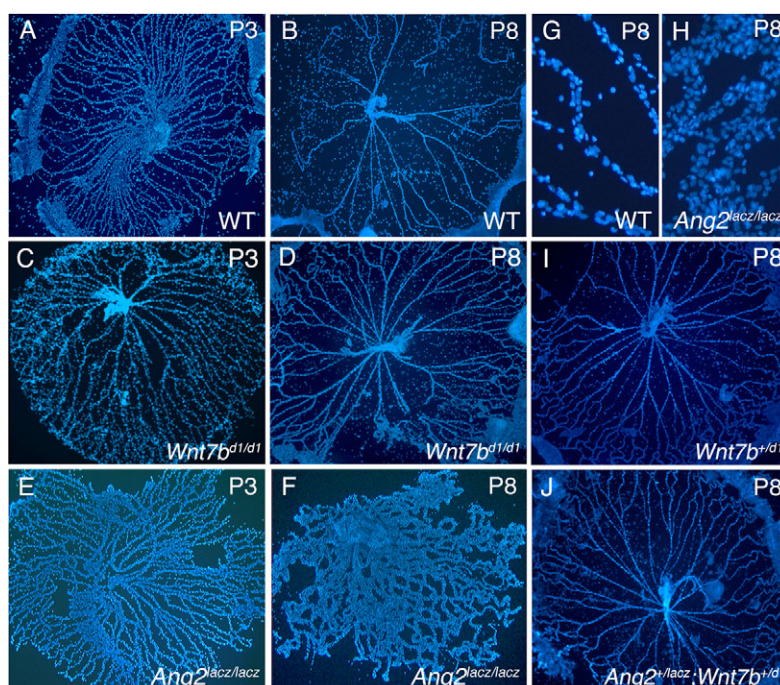
## RESULTS

### Wnt and angiopoietin pathways cooperate to mediate hyaloid vessel regression

We have previously demonstrated that Wnt signaling is important in hyaloid vessel regression (Lobov et al., 2005). However, mechanistically it has been difficult to reconcile how Wnt signaling could culminate in death of VECs. This prompted us to investigate other pathways that might be required. The Tie2 antagonist Ang2 can induce vessel regression in the absence of VEGF, and the Tie2 agonist Ang1 is linked with survival of endothelial cells (Yancopoulos et al., 2000; Lobov et al., 2002). Furthermore, like *Wnt7b<sup>dl</sup>* mutant mice (Lobov et al., 2005) *Ang2* mutants have persistent hyaloid vessels (Gale et al., 2002) suggesting that the Wnt and angiopoietin pathways function in concert to regulate regression. To investigate the nature of this cooperation we assessed genetic interaction between mutant loci representing each pathway [*Wnt7b<sup>dl</sup>* (Lobov et al., 2005) and *Ang2<sup>lacZ</sup>* (Gale et al., 2002)]. By 8 days after birth (P8) in wild-type mice (Fig. 1A,B) hyaloid vessel regression is all but complete. By contrast, *Wnt7b<sup>dl/dl</sup>* (Fig. 1C,D) and *Ang2<sup>lacZ/lacZ</sup>* (Fig. 1E,F) mutants show persistent vessels. Unlike *Wnt7b<sup>dl/dl</sup>* mutant vessels, those from *Ang2<sup>lacZ/lacZ</sup>* mice show an unusual hypercellularity (Fig. 1H). Compound heterozygotes show a persistence phenotype (Fig. 1J) that is more severe than that of either single heterozygote (Fig. 2A) and similar in degree to that of the *Wnt7b<sup>dl</sup>* homozygote (Fig. 2A). Very similar responses are observed when interactions between the Wnt pathway co-receptor gene *Lrp5<sup>lacZ</sup>* and *Ang2<sup>lacZ</sup>* are assessed (data not shown). These data are consistent with a cooperative action between the Wnt and angiopoietin pathways in regulating vessel regression.

### Proliferation and death are exquisitely sensitive to Wnt-Angiopoietin pathway interaction

Evidence for cooperative action of the two pathways also emerges when proliferation and cell death are quantified (Fig. 2B,C). *Ang2<sup>lacZ</sup>* heterozygotes and homozygotes have progressively reduced levels of cell death as determined by TUNEL analysis (Fig. 2B). There is a similar but more pronounced response in *Wnt7b<sup>dl</sup>* mutants (Fig. 2B). Double heterozygotes show a level of cell death significantly less than *Ang2<sup>lacZ</sup>* heterozygotes (Fig. 2B) suggesting that Wnt7b modulates the Ang2 pro-apoptotic function. An examination of BrdU labeling levels is also revealing (Fig. 2C). The *Ang2<sup>lacZ</sup>* heterozygote is essentially unchanged whereas the *Ang2<sup>lacZ</sup>* homozygote has a dramatically increased BrdU labeling index (Fig. 2C), consistent with the distinct hyperplastic phenotype of the hyaloid vessels (Fig. 1H). *Wnt7b<sup>dl</sup>* mutants show the opposite response, with the heterozygote having less than half the wild-type level of labeling (Fig. 2C). Combining *Ang2* heterozygosity with *Wnt7b<sup>+/dl</sup>* completely rescues the proliferation defect in the Wnt mutant. We conclude that for proliferation and cell death, *Wnt7b* and *Ang2* mutations show a genetic interaction and that the pathways are finely balanced in regulating cellular responses.



**Fig. 1. Genetic Interaction between the Wnt and the angiopoietin pathways.** (A–J) Hyaloid vessels from WT (A,B,G), *Wnt7b<sup>d1/d1</sup>* (C,D), *Ang2<sup>lacZ/lacZ</sup>* (E,F,H), *Wnt7b<sup>d1/d1</sup>* (I) and *Ang2<sup>lacZ/lacZ</sup>; Wnt7b<sup>d1/d1</sup>* (J) animals of the indicated ages stained with Hoechst 33258. (H) Hypercellularity of *Ang2<sup>lacZ/lacZ</sup>* hyaloid capillaries compared with WT (G) at P8. Magnifications: 50× in A–F,I,J; 400× in G,H.

### The Akt component of Tie2 signaling is critical for cell survival

*Wnt7b* is expressed by hyaloid-associated macrophages (Lobov et al., 2005). X-Gal staining of hyaloid vessels from *Ang2<sup>lacZ/lacZ</sup>* mice revealed that *Ang2* is expressed in capillary cells (Fig. 3A). The X-Gal staining pattern (Fig. 3B, red arrowheads) was characteristic of pericyte processes. Resident macrophages (Fig. 3B, red dashed rings) did not stain. Small-diameter capillaries that typically have minimal pericyte investment also had limited staining (Fig. 3B, red bracket). Immunofluorescent staining of hyaloid vessels from wild-type mice with desmin, a marker for pericytes, also showed a similar pattern of staining as the X-Gal-positive cells (Fig. 3C, white arrowheads). X-Gal staining of hyaloid vessels from *Tie2-lacZ* mice (Schlaeger et al., 1997) showed a contrasting pattern of labeling similar to that of VECs (Fig. 3D). This suggested that the source of *Ang2* required for hyaloid vessel regression was the pericyte.

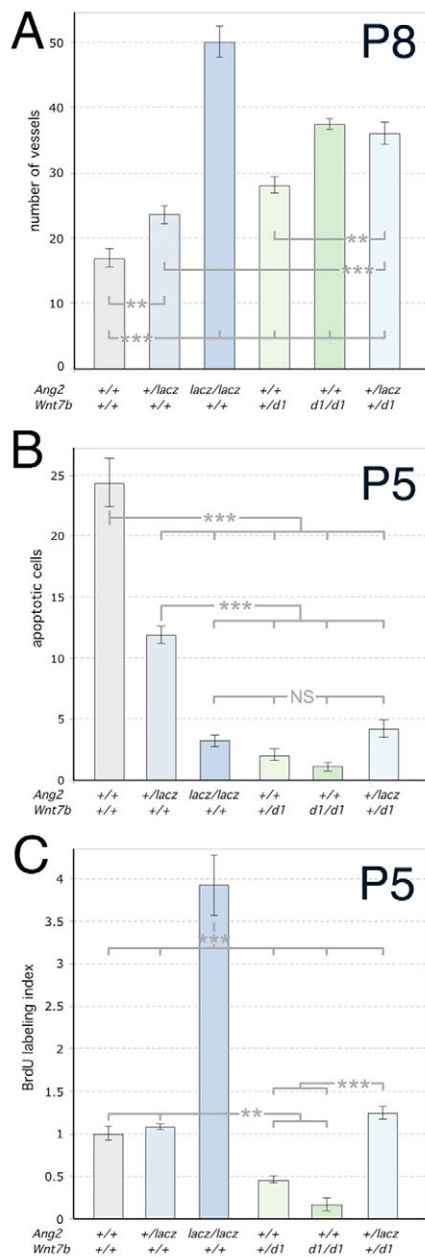
The angiopoietin pathway incorporates the serine-threonine kinase Akt (Kim et al., 2000a). Akt is known to have multiple effects, including suppression of cell death (Datta et al., 1999) and in some settings, stabilization of  $\beta$ -catenin (Moore and Lemischka, 2006). Since *Ang2* is an antagonist, loss-of-function *Ang2* should be equivalent to gain-of-function Akt. To determine whether this was the case, we used the TET transactivation system (Corbel and Rossi, 2002) to express a dominant active form of Akt in VECs. When tetracycline is withdrawn, bitransgenic *VE-cadherin:tTa;TET:myrAkt* mice express *myrAkt* in VECs (Sun et al., 2005). Withdrawal of tetracycline at the day of birth followed by BrdU labeling at P5 showed that Akt gain-of-function mice have an increased labeling index (Fig. 3J, light green bar) that is comparable to that obtained with the intra-vitreous injection of *Ang1* (an alternative strategy for pathway activation; Fig. 3J, green bar) or

when the *Ang2* gene is mutated (Fig. 3J, red bar). These data indicate that activation of the angiopoietin pathway stimulates proliferation, that proliferation responses occur downstream of Akt, and confirms that *Ang1* and *Ang2* behave as agonist and antagonist, respectively.

Akt gain-of-function phenocopies other aspects of the *Ang2<sup>lacZ/lacZ</sup>* hyaloid persistence phenotype (Fig. 3E–L). In *VE-cadherin:tTa;TET:myrAkt* mice, levels of apoptosis are low at P5 (Fig. 3K), vessel number is greater at P8 (Fig. 3L) and although there is some variability (Fig. 3F,G) many vessels have the hypercellularity (Fig. 3I) characteristic of the *Ang2* mutant (Fig. 1H). Limited stability of *Ang1* after intra-vitreous injection precluded assessment of vessel number at P8. To perform Akt loss-of-function experiments, we took advantage of the Akt inhibitor SH6 (Kozikowski et al., 2003; Castillo et al., 2004) and compared the consequences of injecting *Ang1*, SH6 or both, into the vitreous of wild-type mice at P4. Hyaloid vessels were dissected at P5 and TUNEL analysis performed (Fig. 3M,N) so that we could quantify the total remaining healthy capillaries (Fig. 3O). SH6 injection resulted in a dramatic increase in cell death and precocious hyaloid regression (Fig. 3M–O). Importantly, the specificity of the SH6 cell death response was demonstrated by the rescue of precocious regression when *Ang1* was co-injected (Fig. 3O). The ability of *Ang1* to reverse SH6-induced cell death may indicate that the inhibitor is in equilibrium with Akt and can be competed out if sufficient activated Akt is provided by *Ang1* stimulation. Combined, these data indicate that Akt is a key mediator of both proliferation and survival responses in VECs of the hyaloid system.

Stabilization of  $\beta$ -catenin via Akt has been documented in other systems (Fukumoto et al., 2001; Sharma et al., 2002) and this was one possible explanation for the cooperative action of Wnt and





**Fig. 2. The Wnt and angiopoietin pathways are finely balanced in regulating cell death, proliferation and hyaloid regression.** (A–C) Numbers of hyaloid vessels at P8 (A), apoptotic cells at P5 (B) and BrdU-labeled cells at P5 (C) in animals of the indicated genotypes. Significance was assessed using one-way ANOVA. All genotypes were compared to WT and were significantly different as indicated by the asterisks. Brackets indicate the groups that were compared using one-way ANOVA. Significance levels: \*\* $P \leq 0.01$ ,  $>0.001$ ; \*\*\* $P \leq 0.001$ ,  $>0$ .

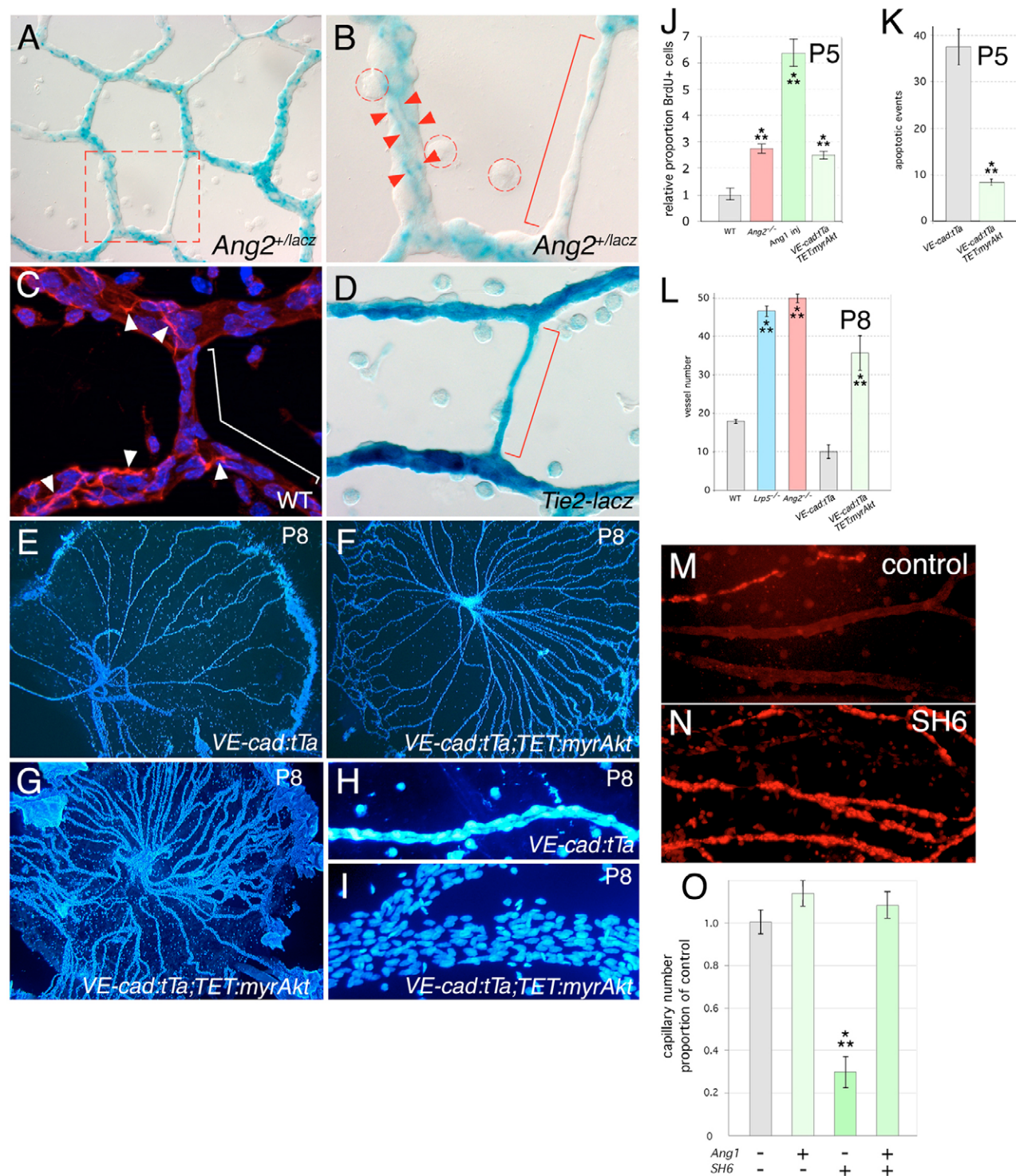
angiopoietin pathways during hyaloid regression. We examined this possibility in a culture system using  $\beta$ -catenin labeling of microvascular endothelial cells (MVECs) under various treatment conditions. MVECs were serum-starved overnight to remove the influence of other agents, and then incubated for 12 hours (Fig. 4A–F) with serum-free medium as a control (Fig. 4A), with recombinant Wnt3a (Fig. 4B) or with Ang1 (Fig. 4C). Whereas there were changes in the strength of  $\beta$ -catenin labeling in other regions of the

cells, Ang1, like Wnt3a, was found to stimulate translocation of  $\beta$ -catenin to the nucleus. Prevention of nuclear  $\beta$ -catenin localization, when wortmannin (a PI 3-kinase inhibitor; Fig. 4D) or SH6 (an Akt inhibitor; Fig. 4E) were added in combination with Ang1, provided evidence that  $\beta$ -catenin stabilization was downstream of PI 3-kinase and Akt. Quantification also showed that at the concentrations of recombinant ligands chosen, Ang1 was at least as potent as Wnt3a in stimulating this response (Fig. 4F). Furthermore, the inability of wortmannin or SH6 to prevent  $\beta$ -catenin nuclear localization in response to Wnt3a emphasizes that the Wnt and angiopoietin pathway are alternative  $\beta$ -catenin stabilization options (Fig. 4F). We also performed experiments in which MVECs were incubated with Ang1, Wnt3a or both for 4 hours (Fig. 4F, right panel). Since the combined factors gave a synergistic response, this also suggested the Wnt and angiopoietin pathways cooperate to stabilize  $\beta$ -catenin.

To determine whether  $\beta$ -catenin might be stabilized via the angiopoietin pathway in vivo, we performed  $\beta$ -catenin immunolabeling on hyaloid preparations from various mouse mutants.  $\beta$ -catenin associated with junctional complexes in wild-type hyaloid vessels is abundant (Fig. 5A). By contrast, the nuclear  $\beta$ -catenin indicative of normal Wnt pathway signaling is difficult to detect. To improve our ability to detect nuclear  $\beta$ -catenin, we developed a labeling protocol in which we performed two rounds of primary antibody incubation and detected each round with color-differentiated secondary antibodies (Fig. 5A–L). This may work more effectively than a single round of primary antibody because the first round saturates abundant junctional  $\beta$ -catenin epitopes. Thus, as expected,  $\beta$ -catenin levels in wild-type (Fig. 5A–C) and *Lrp5* mutant (Fig. 5D–F) hyaloid vessels were high at junctions but nuclei were not obviously positive. By contrast, in both the *Ang2* homozygote (Fig. 5G–I) and the myrAkt gain-of-function mice (Fig. 5J–L) nuclear  $\beta$ -catenin signal was robust. The response was somewhat patchy in the Akt gain-of-function mice, perhaps due to non-uniform gene expression induced by tetracycline withdrawal. Junctional  $\beta$ -catenin was downregulated with Akt gain of function (Fig. 5K,L). These observations indicate that in VECs, Akt activates  $\beta$ -catenin.

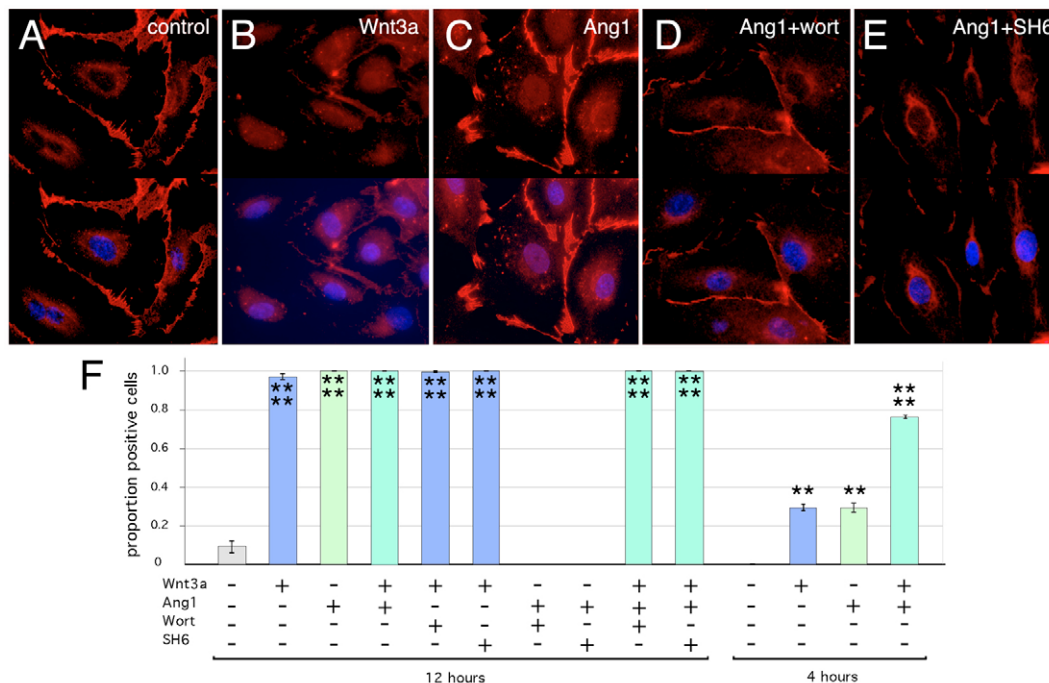
As an alternative way to assess angiopoietin-regulated Wnt pathway responses, we quantified *TOPGAL* reporter expression in the hyaloid vessels when Ang1 or 2 was injected into the eye. Previously we validated the *TOPGAL* transgene – a *lacZ* open reading frame regulated by Lef/TCF binding sites – as a Wnt pathway reporter for VECs of the hyaloid capillaries (Lobov et al., 2005). We have also demonstrated that *lacZ* expression and X-Gal staining in *Lrp5*<sup>lacZ/lacZ</sup> mice is very weak in all VECs, and therefore can be easily distinguished from the strong, sporadic VEC staining of the *TOPGAL* transgene (Lobov et al., 2005). Intra-vitreous injection of recombinant Ang1 increased the number of *TOPGAL*-expressing cells regardless of the *Lrp5* status of the experimental mouse (Fig. 5M–O). This indicates that the angiopoietin pathway can activate a model Wnt pathway target gene and is consistent with Akt-mediated stabilization of  $\beta$ -catenin.

Injection of Ang2 into *TOPGAL* mice gave differing responses depending on *Lrp5* status. In *Lrp5* homozygous mutants, *TOPGAL*-positive cell number was close to zero and, as might be anticipated from an antagonist, injection of Ang2 did not elicit a change (Fig. 5P). In *Lrp5* heterozygous mutants also, as expected, Ang2 reduced the number of *TOPGAL*-expressing cells (Fig. 5P). Surprisingly, in wild-type mice, Ang2 injection reproducibly elicited the opposite response and increased the number of *TOPGAL*-expressing cells (Fig. 5P). In seeking an explanation for this, we considered the possibility that besides regulating angiopoietin pathway signaling,



**Fig. 3. Angiopoietin pathway responses downstream of Akt regulate hyaloid regression.** (A,B) X-Gal staining of P5 hyaloid vessels from *Ang2<sup>lacZ/lacZ</sup>* mice. Dashed red box in A indicates region shown at higher magnification in B. Red arrowheads indicate X-Gal-labeled cell processes, the red bracket indicates a small capillary with limited X-Gal staining. Macrophages are indicated by the dashed red circles. (C) Pericytes from P5 hyaloid vessels of wild-type mice labeled with desmin (red). Nuclei are stained with Hoechst 33258 (blue). White arrowheads indicate cell processes of the pericytes, the white bracket indicates areas with no desmin staining. (D) X-Gal staining of P5 hyaloid vessels from a *Tie2-lacZ* mouse in which *lacZ* is expressed in vascular endothelial cells (VECs). The red bracket indicates a small capillary with uniform X-Gal staining. (E-I) Hoechst 33258-labeled hyaloid vessels from control (E,H) and *VE-cadherin:ΔTET:myrAkt* (F,G,I) transgenic animals. (J) Proportion of BrdU-positive cells in P5 hyaloid vessels of WT, *Ang2<sup>lacZ/lacZ</sup>*, WT injected with Ang1 and *VE-cadherin:ΔTET:myrAkt* mice. (K) Number of apoptotic events at P5 in the hyaloid vessels from mice of the indicated genotypes. (L) Hyaloid vessel number at P8 in mice of the indicated genotypes. (M,N) TUNEL labeling of hyaloid vessel preparations with no treatment (M) or after intravitreal injection of SH6 (N). (O) Quantification of healthy hyaloid vessel number as a proportion of wild type after injection of the indicated reagents at P4 and assessment at P5. Magnifications: 50× (E-G), 200× (A), 400× (M,N), 630× (B,C,D,H,I). Significance levels: \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , >0.





**Fig. 4. The Tie2 and canonical Wnt pathways are integrated through Akt.** (A-E) Immunofluorescence detection of  $\beta$ -catenin in primary MVECs either untreated (A) or treated with Wnt3a (B), Ang1 (C), Ang1 + wortmannin (D) or Ang1 + SH6 (E). Upper and lower parts of each panel show  $\beta$ -catenin labeling alone and merged nuclear and  $\beta$ -catenin labeling, respectively. (F) Proportion of MVECs with nuclear  $\beta$ -catenin detectable by immunofluorescence under the conditions indicated. For this quantification, the significance levels are: \*\* $P < 0.005$ , \*\*\* $P < 0.00005$ .

Ang2 might have an additional activity regulating macrophage production of Wnt7b. Using the *Wnt7b<sup>lacZ</sup>* allele, we assessed gene expression on the *Ang2<sup>lacZ</sup>* heterozygous and homozygous backgrounds and found that when Ang2 activity was absent, *Wnt7b* expression in macrophages was lost (Fig. 6A,B). Since it could be argued that macrophages in the *Ang2<sup>lacZ</sup>* null might be poorly differentiated and therefore incapable of expressing *Wnt7b*, we also performed a gain-of-function experiment. Ang2 was injected intra-vitally in CD1 animals, hyaloid macrophages were isolated by laser capture microdissection and *Wnt7b* RT-PCR performed (Fig. 6C). Though *Wnt7b* expression in the control eye showed some variation, from being undetectable to being weakly present, in every experiment Ang2 injection resulted in upregulation of *Wnt7b*. These data show that directly or indirectly, Ang2 is required for *Wnt7b* expression in macrophages. This was a straightforward explanation for the increased number of cells expressing *TOPGAL* with injection of Ang2. Combined with the above analysis, this suggests that Ang2 has two functions, the pro-apoptotic suppression of Akt and the pro-cycle activation of macrophage *Wnt7b*. Expression of Ang1 in the hyaloid vessel complex (Fig. 6D) suggested that Ang1 and Ang2 had the opportunity to function as agonist and antagonist.

### Cell cycle entry is required for hyaloid VEC apoptosis

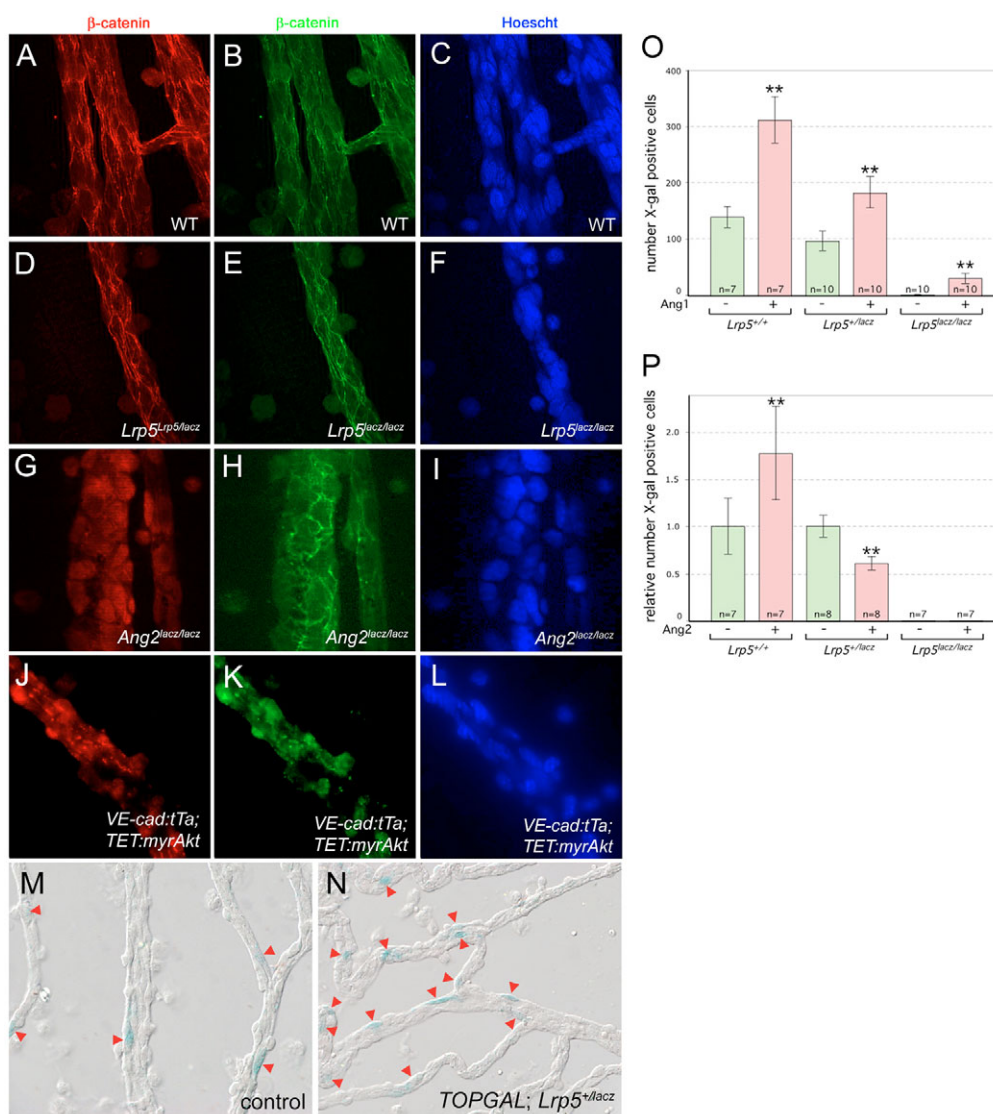
Cell death is sometimes coupled to cell cycle progression (Diez-Roux et al., 1999; Lowe et al., 2004). This strategy of cell death regulation could explain why *Wnt7b* – a promoter of cell cycle entry in hyaloid VECs (Lobov et al., 2005) – is required for cell death. To address the relationship between cell cycle entry and cell death directly, we used BrdU labeling to track the fate of a synchronous cohort of hyaloid VECs through the cell cycle. BrdU was injected at

$t=0$  hours and hyaloid vessel preparations fixed at  $t=1$  hour, then at 4-hour intervals subsequently. We scored the number of cells that had both TUNEL and BrdU labeling (Fig. 6E,F). This revealed that BrdU-labeled, apoptotic cells rose to a peak, 17 hours after the BrdU injection (Fig. 6I, red line). The existence of this peak indicates that cell death is dependent on cell cycle stage. By scoring the occurrence of paired, BrdU-labeled nuclei that are the products of cell division (Fig. 6G,H), we were also able to define when, on average, the cohort of BrdU-labeled cells left M phase and entered G1 phase. The peak of G1 phase daughter cells corresponded with the peak of BrdU-positive, apoptotic cells at 17 hours after BrdU (Fig. 6J, red line). These data (confirmed in two independent experiments) indicate that cell cycle progression and cell death are coupled, and that cells die from G1 phase of the cell cycle.

To examine the role of the angiopoietin pathway in regulating cell cycle stage-dependent cell death we injected Ang1 intra-vitally into the right eye of each animal 6 hours after the BrdU injection. This resulted in the absence of a cell death peak at 17 hours (Fig. 6I, blue line) but did not significantly influence the appearance of daughter pairs (Fig. 6J, blue line). These data indicate that Ang1 suppresses cell death and that the effects of this are observed in the G1 phase of the cell cycle. Combined with previous work (Lobov et al., 2005) these experiments show that the Wnt and angiopoietin pathways are integrated through the cell cycle – *Wnt7b* promotes cell cycle entry and Ang2 promotes cell death from G1 phase.

### DISCUSSION

In the current study we sought to explain how the Wnt and the angiopoietin pathways cooperate to regulate scheduled vascular regression. We have shown that there are multiple levels of pathway integration that constitute an Ang2-mediated cell death switch (Fig. 7).



**Fig. 5. The angiopoietin pathway activates Wnt target genes via Akt and  $\beta$ -catenin.** (A-L) Immunofluorescent staining for  $\beta$ -catenin (green and red with two rounds of labeling – see text) and nuclei (blue) in hyaloid preparations in mice of the indicated genotypes. (M,N) X-Gal staining of hyaloid vessels (red arrowheads) from *TOPGAL; Lrp5*<sup>+/lacZ</sup> animals that were (M) mock injected intra-vitreally or (N) injected with Ang1 intra-vitreally. (O,P) Quantification of X-Gal-stained cell numbers (as in M and N) from hyaloid vessels of the indicated genotypes injected with Ang1 (O) or Ang2 (P). Significance levels: \*\* $P \leq 0.01$ ,  $> 0.001$ .

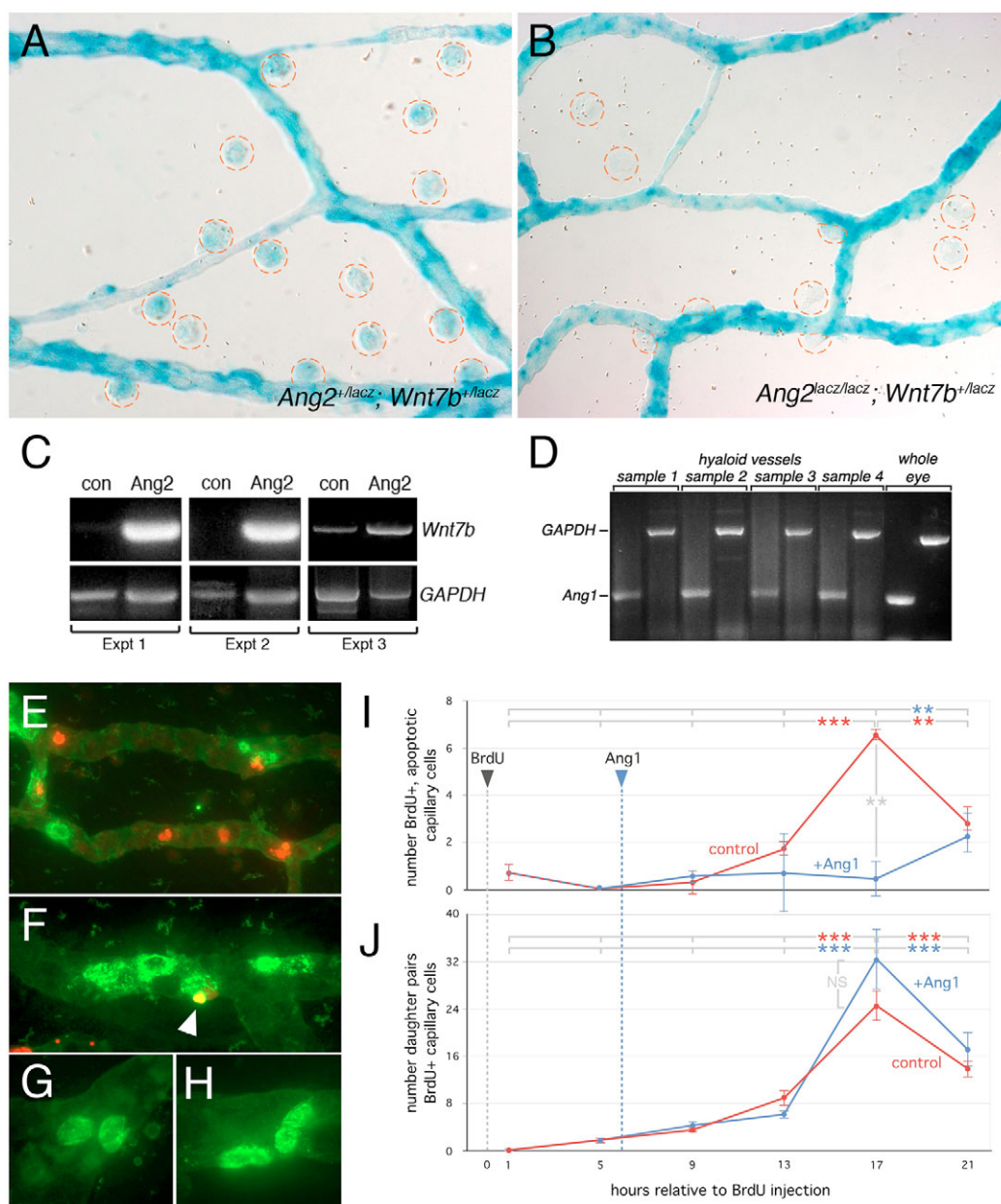
### Mechanism of the cell death switch

Current data indicate that cell cycle entry is a crucial element of the switch because it is a precondition for cell death, an observation consistent with coupling between cell cycle progression and cell death in other systems (Lowe et al., 2004). In the hyaloid vessels, Wnt pathway activation is a stimulus for cell cycle entry and essential for cell death (Lobov et al., 2005). An explanation for the Wnt pathway requirement emerges from the demonstration that hyaloid VECs die from G1 phase of the cell cycle when Ang2 suppresses survival signaling (Fig. 6).

Much remains unknown about the mechanism of cell cycle-cell death coupling. It has been observed that in general, cells are more sensitive to death stimuli when they are actively cycling. One mechanism for coupling proliferation and death appears to be the ability of growth promoting proteins such as Myc [a Wnt pathway target (He et al., 1998)] to sensitize cells to pro-apoptotic stimuli (including survival signal withdrawal) through enhanced

cytochrome C release and caspase 9 activation (Evan and Vousden, 2001). Furthermore, in many settings where proliferating cells die, they do so at cell cycle checkpoints. The p53 genotoxic damage control checkpoint (Sherr and Roberts, 1995) and the so-called Restriction Point (Pardee, 1989; Planas-Silva and Weinberg, 1997) at which survival factors are critical (Zhu et al., 1996; Assoian, 1997; Planas-Silva and Weinberg, 1997) can result in apoptosis in G1 phase of the cell cycle.

A hyaloid VEC will survive (death switch in the off position, Fig. 7A) when Ang1 is the predominant influence. This activates Akt and suppresses cell death, presumably through the well-described inhibition of the pro-apoptotic mediators FoxO and Bad (Datta et al., 1999). Our data also indicate that activation of the angiopoietin pathway is accompanied by proliferation. In this system, proliferation is at least partly a consequence of Akt-mediated stabilization and nuclear translocation of  $\beta$ -catenin (Fig. 7A).

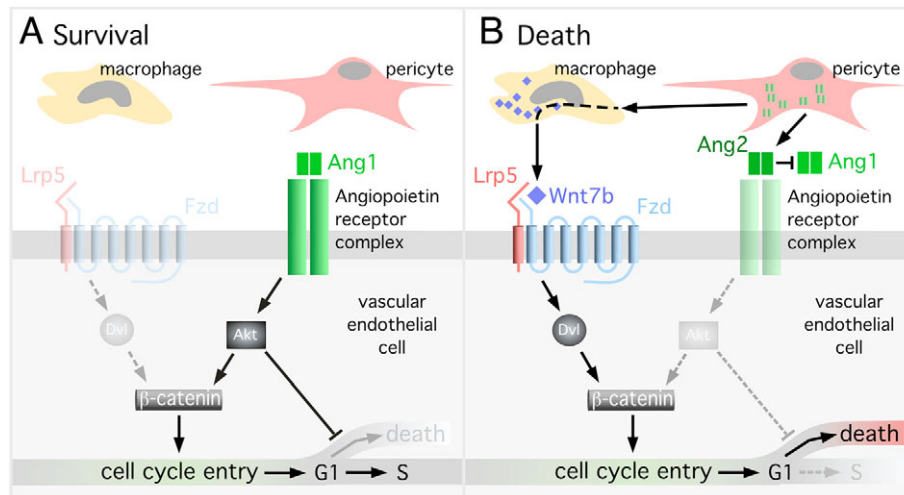


**Fig. 6. Angiopoietin signaling regulates *Wnt7b* expression and cell cycle stage-dependent cell death.** (A,B) X-Gal staining of hyaloid vessels from *Ang2<sup>lacZ/+</sup>; Wnt7b<sup>lacZ/+</sup>* (A) and *Ang2<sup>lacZ/lacZ</sup>; Wnt7b<sup>lacZ/+</sup>* (B) animals. Macrophages are circled in red. (C) *Wnt7b* and *Gapdh* RT-PCR of laser captured macrophages from hyaloid vessels of P3 wild-type animals, injected intra-vitreally with Ang2 (Ang2) or sham injected (con). Data from three independent experiments are presented. (D) *Ang1* and *Gapdh* RT-PCR from wild-type P5 hyaloid vessel mRNA. Whole eye RNA was used as a control. (E-H) Combined BrdU (green) and TUNEL (red) labeling of hyaloid vessels showing BrdU-positive VECs that are apoptotic (F, indicated by white arrowhead) and pairs of BrdU-positive daughter nuclei (G,H). (I,J) BrdU labeled cell fate mapping: red lines show the number of BrdU-TUNEL double labeled nuclei (I) and BrdU-positive daughter pairs (J) in left eye hyaloid vessels assessed over a 21 hour time-course after BrdU incorporation. The blue lines in I and J show similar quantification in the right, contralateral eye after Ang1 injection at  $t \approx 6$  hours. Significance levels:  $**P \leq 0.01$ ,  $>0.001$ ;  $***P \leq 0.001$ ,  $>0$ .

When the death switch is in the on position (Fig. 7B) Ang2 has two functions. The first function is to suppress Akt activity and promote cell death (Datta et al., 1999). However, in suppressing Akt, Ang2 also eliminates the cell cycle entry stimulus (via  $\beta$ -catenin) that is a prerequisite for cell death. Thus, the second function of Ang2, which is upregulation of macrophage *Wnt7b* expression, appears to be an adaptation to provide an alternative pathway to stimulate cell cycle

entry and coupled cell death. How Ang2 upregulates *Wnt7b* expression in macrophages is currently unclear. This could be an indirect consequence of VECs producing a signaling molecule in response to Ang2 ligation of Tie2. Alternatively, macrophages might respond directly to the presence of Ang2 through the integrins that represent alternative receptors for the angiopoietins (Carlson et al., 2001; Camenisch et al., 2002; Cascone et al., 2005).





**Fig. 7. Model of the angiopoietin 2-dependent cell death switch.** (A) Hyaloid VECs will survive when Ang1 activates Akt, suppresses cell death and promotes proliferation through  $\beta$ -catenin stabilization. (B) Hyaloid VECs will die when Ang2 promotes cell death via suppression of Akt and cell cycle entry by upregulating Wnt7b in macrophages. In the absence of survival signals the cycling hyaloid VECs will undergo apoptosis in the G1 phase of the cell cycle.

### Macrophage function in homeostasis and disease

In his phagocytosis theory, Nobelist Ilya Metchnikoff first formulated the idea that macrophages could ‘police’ the tissues as a way of reconciling inherent chaos (Tauber, 2003). Indeed, the ability of macrophages to identify, engulf and dispose of foreign matter and apoptotic cells gives them a crucial role in tissue homeostasis. Even a modest reduction in this function can result in the chronic inflammation that promotes diseases such as cancer and diabetes (Wellen and Hotamisligil, 2005; Condeelis and Pollard, 2006). Being widely distributed and highly mobile, macrophages are perfectly suited to this homeostatic function.

In a few documented circumstances, macrophages take on the additional role of actively signaling apoptosis in a target cell. A number of macrophage products have been implicated: nerve growth factor (NGF) where macrophage-related microglia cause neurons to die in the chick (Frade et al., 1996) and superoxide ions where microglia signal apoptosis of Purkinje cells in mouse brain slice cultures (Marin-Teva et al., 2004; Mallat et al., 2005). An additional example – where the dead cell recognition machinery is a back-up pathway for caspase pathway activation in *C. elegans* – is important because it implies that phagocyte-induced cell death is a highly conserved activity that will probably be found throughout the metazoans (Hoeppner et al., 2001; Reddien et al., 2001).

In the current work we describe a cell death signaling mechanism that involves three cell types, the pericyte, the macrophage and the vascular endothelial cell. Ang2, which is probably produced by pericytes, is a critical initiator of a switch in the pattern of signaling that results in VEC cell cycle entry and cell death (Fig. 7). In this system, the macrophage is the critical source of Wnt7b (Lobov et al., 2005) that is required for VEC cell cycle entry. In turn, Wnt7b expression in macrophages requires Ang2. This arrangement of regulatory interactions means that participation of the macrophage is obligatory. If the macrophage is absent, there would be no source of Wnt7b to stimulate cell cycle entry, and no cell death. Ang2-dependent Wnt7b expression may represent the optimal way to ensure that when cell death occurs the macrophage and its dead cell recognition and disposal capabilities are present. We anticipate that this or related mechanisms may be generally applicable.

Macrophages have been implicated in a variety of diseases, often where angiogenesis is a prominent component of the disease process. One example is the vision-compromising choroidal neovascularization that occurs in the wet form of age-related macular degeneration (AMD) (Ferrara and Kerbel, 2005). In this

setting, according to depletion experiments (Espinosa-Heidmann et al., 2003; Sakurai et al., 2003) macrophages enhance the formation of new blood vessels. Another example is the activity of tumor-associated macrophages (TAMs) in enhancing tumor angiogenesis and tumor progression (Condeelis and Pollard, 2006). In a mouse breast cancer model that closely mimics the human disease (Lin et al., 2003), macrophage deficiency leads to tumors that are poorly vascularized and in a response that is probably related, essentially non-metastatic (Lin et al., 2006).

In diseases like AMD and cancer where macrophages play a prominent role, it will be interesting to determine whether macrophage production of Wnt ligands is a factor contributing to disease progression. Based on the current work, macrophage Wnts are a stimulus for VEC proliferation and in the absence of a second, modulating signal (Ang2 in the hyaloid system, Fig. 7) could stimulate angiogenesis and promote disease progression. Further work will be needed to address these questions.

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